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Review

Osteosarcoma: Molecular Pathogenesis and iPSC Modeling

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Rare hereditary disorders provide unequivocal evidence of the importance of genes in human disease pathogenesis. Familial syndromes that predispose to osteosarcomagenesis are invaluable in understanding the underlying genetics of this malignancy. Recently, patient-derived induced pluripotent stem cells (iPSCs) have been successfully utilized to model Li–Fraumeni syndrome (LFS)-associated bone malignancy, demonstrating that iPSCs can serve as an *in vitro* disease model to elucidate osteosarcoma etiology. We provide here an overview of osteosarcoma predisposition syndromes and review recently established iPSC disease models for these familial syndromes. Merging molecular information gathered from these models with the current knowledge of osteosarcoma biology will help us to gain a deeper understanding of the pathological mechanisms underlying osteosarcomagenesis and will potentially aid in the development of future patient therapies.

Population at Risk for Osteosarcoma

Bone cancer is one of the most common primary malignancies in children and adolescents. Osteosarcoma comprises almost 60% of the common histological subtypes of pediatric bone sarcoma. It most frequently arises in the **epiphysis** (see Glossary) of long bones [1]. Standard treatment for osteosarcoma consists of surgery and chemotherapy. While the 5 year survival rate of non-metastatic disease hovers at ~70%, metastatic disease – most often to the lungs – is associated with survival rates of 15–30% [1]. Osteosarcoma predominantly affects children and adolescents between the ages of 5 and 20 years, as well as adults in their seventies, with approximately 400 new pediatric cases diagnosed in the USA annually [1].

Despite advances in surgery and multiagent chemotherapy, lack of understanding of the molecular mechanisms of osteosarcomagenesis has prevented significant improvement in the survival of patients over the past 40 years. This malignancy makes osteosarcoma one of the leading causes of cancer mortality among children and adolescents. Therefore, elucidation of individual osteosarcoma-associated gene functions to explore the possible pathological mechanisms involved in osteosarcoma initiation, development. and progression is crucial for future osteosarcoma detection and treatment. People with some rare hereditary genetic disorders are particularly at high risk for developing osteosarcoma. These include patients with Li–Fraumeni syndrome (LFS), hereditary retinoblastoma (RB), Rothmund–Thomson syndrome (RTS), RAPADILINO syndrome (RAPA), Werner syndrome (WS), Bloom syndrome (BS), Diamond–Blackfan anemia (DBA), and Paget's disease of bone (PDB) (Boxes 1–5). Importantly, alterations in these genetic disease-associated genes have also been identified

Trends

Osteosarcoma can be derived from undifferentiated/dedifferentiated mesenchymal stem cells and osteoblast-committed cells with differentiation defects.

Other than mutations, genomic rearrangements are also involved in osteosarcomagenesis, which may be ignored by traditional mutation analysis.

Osteosarcoma-specific fusion genes offer potential therapeutic targets for further osteosarcoma treatment.

Insights gained from osteosarcoma-prone diseases highlight numerous interesting concepts linked to cancer development, including differentiation control, tumor-associated immunosuppression, and autophagy.

Several osteosarcoma-prone iPSC disease models have been established, including Li–Fraumeni syndrome, hereditary retinoblastoma, Werner syndrome, and Diamond–Blackfan anemia. These systems provide a new platform for modeling and investigating the pathogenesis of osteosarcoma.

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Box 1. Li–Fraumeni Syndrome (LFS)

LFS is a rare hereditary autosomal dominant cancer disease first identified by Drs F.P. Li and J.F. Fraumeni, Jr in 1969 [127]. They studied four families with soft-tissue sarcomas and found a high rate of familial tumor aggregation that could not be explained by chance alone. They further described this disease as a genetically heterogeneous inherited syndrome of pediatric soft-tissue sarcomas, breast cancers, and other neoplasms in young adults [127]. Its autosomal dominant inheritance pattern was confirmed in 1988 [128]. Patients with LFS are likely to develop a primary cancer diagnosis by 20 years of age and secondary malignancies are common. Germline mutations in *TP53* were found to be responsible for most cases of LFS in 1990 [129]. In contrast to other inherited cancer syndromes largely characterized by tissue and site specificity, LFS patients present with a variety of early-onset primary tumors, including soft tissue sarcomas and osteosarcomas, breast cancers, brain tumors, leukemias, and adrenocortical carcinomas [128]. The clinical criteria for classic LFS are based on meeting the following three criteria: (i) a sarcoma diagnosed before the age of 45 years, (ii) a first-degree relative with any cancer before the age of 45 years, and (iii) a first- or second-degree relative with any cancer before the age of 45 years or a sarcoma at any age. In addition, some patients who do not meet the classic LFS criteria nevertheless harbor germline *TP53* mutations, which leads to the less-stringent Chompret criteria to identify families at risk for LFS and thus eligible for *TP53* testing [130]. LFS-associated germline *TP53* mutations are relatively rare, occurring in 1 in 20,000 individuals [131]. Over 300 distinct *TP53* mutations causing LFS have been identified (http://p53.free.fr/Database/p53_cancer_db.html). Approximately 70% of these mutations occur in the DNA-binding domain encoded by exons 5–8; over 75% are missense mutations and usually generate a truncated p53 protein (http://p53.free.fr/Database/p53_cancer_db.html). The top mutational hotspots include R175, Y220, G245, R248, R273, R282, and R337 [22]. All these *TP53* mutations except R337H – a founder mutation in Southern and Southeastern Brazilian populations –are also widely found in sporadic cancers [22]. Commonly, *TP53* mutations not only abolish p53 normal function but have also been associated with gain of oncogenic function [22,30,31].

in samples of human sporadic osteosarcoma, accounting for the vast majority of osteosarcoma cases in the general population. As new information has emerged, so has the possibility of modeling osteosarcoma biology, and one approach has included the use of patient-derived **induced pluripotent stem cells** (iPSCs), as in the case of LFS-associated bone malignancy. Indeed, investigations into inherited osteosarcoma-associated gene functions, coupled to iPSC modeling strategies, will undoubtedly provide valuable insight into better understanding bone tumor initiation, development, progression, and treatment.

Genetics of Osteosarcoma

Osteosarcoma frequently carries gross genomic mutations and rearrangements including chromosomal translocations [2–4]. **Chromothripsis** – the presence of thousands of clustered chromosomal rearrangements – is observed in 25% of clinical osteosarcoma human samples compared to 2–3% of cancers overall, such as chronic lymphocytic leukemia (CLL) [4]. Chromosomal translocations and mutations may juxtapose proto-oncogenes with constitutively active promoters, cause deletion of tumor-suppressor genes, or produce chimeric oncogenes (e.g., *PMP22–ELOVL5*) [2–5]. Germline and somatic genome sequencing efforts have revealed potential pathological mechanisms involved in both osteosarcoma and syndromes with a genetic predisposition to osteosarcoma. For instance, the St Jude Children's Research Hospital – Washington University Pediatric Cancer Genome Project compared the results of **whole-genome sequencing** (WGS) from osteosarcoma specimens with matched germline DNA from affected patients and identified high rates of **structural variations** (SVs) and **copy-number alterations** (CNAs), but low rates of **single-nucleotide variations** (SNVs) in osteosarcoma tumors [2], indicating that chromosomal lesions by SVs and CNAs, rather than SNVs, are the main mechanism of recurrent mutations in osteosarcoma. Consistent with this, a study of genomic alterations in pediatric cancers indicated that osteosarcomas exhibit the highest frequency of SVs among all pediatric cancers [6]. The genes *TP53*, *RB1*, *ATRX*, and *DLG2* are altered via SVs and/or SNVs with high frequency in osteosarcoma [2]. Inactivation of the tumor-suppressor p53 by translocation into the first intron of the *TP53* gene has been detected in 9 of 19 patient osteosarcoma tumors [2]. Although SNVs in the osteosarcoma genome are relatively uncommon, both SVs and SNVs can result in inactivating mutations in the p53 pathway, a feature found in ~95% of osteosarcomas [2]. Of note, **kataegis**, a pattern of

Box 2. Hereditary Retinoblastoma (RB)

RB, caused by autosomal dominant mutations in the *RB1* tumor-suppressor gene, is a rare cancer of the retina typically found in children, with ~300 cases in the USA and 5000–10 000 cases worldwide reported annually [132]. The RB autosomal dominant inheritance pattern suggests that one copy of the *RB1* mutation in each cell is sufficient to increase patient RB risk [132]. Patients with bilateral RB are likely to acquire *RB1* germline mutations. By contrast, patients with unilateral retinoblastoma usually harbor sporadic *RB1* mutations. Clinical presentation suggests the incidence of unilateral retinoblastoma is 1.5-fold higher than that of bilateral retinoblastoma [133]. Although RB is one of the most treatable pediatric cancers, with 95% 5 year actuarial survival rates in the USA [134], RB patients are prone to develop a second cancer, including sarcomas, melanomas, or brain cancers [135]. Osteosarcoma is the leading cause of death in RB survivors [136]. Receiving radiation therapy and systemic chemotherapy are important risk factors for developing osteosarcoma in RB patients [137].

One study indicated that, among 848 radiation therapy-treated RB patients, 188 patients developed second cancers, 70 of which were osteosarcoma [136], consistent with other reported rates [135]. Somatic mutations of *RB1* are also found in 30–75% of sporadic osteosarcomas [116,122,138]. Most of these osteosarcomas have been found to consist of poorly differentiated cells based on histological data [139], implying that fully functional RB1 might be crucial for normal osteoblast development [16,43]. Interestingly, conditional osteoblast-specific *Rb1* knockout mice exhibit defects in bone development and have increased osteoprogenitors in **calvaria** [140]; these osteoprogenitors might differentiate into immature osteoblasts and subsequently develop into a malignant osteosarcoma following additional cellular events (e.g., gain of somatic mutations and/or dysregulation of signaling pathways). This suggests that Rb1/RB1 might be able to control early osteoblast progression, and its dysregulation may increase the risk of osteosarcoma development; however, further studies will be necessary to validate this concept.

localized hypermutations caused by SNVs, has also been widely found throughout the human osteosarcoma genome [2]. These results offer insight into novel genes that may contribute to the molecular pathogenesis of osteosarcoma and emphasize the value of comprehensive WGS in investigating the genetic features of osteosarcoma.

Hundreds of genomic rearrangements have been identified in osteosarcomas using genomic and transcriptomic analysis, including recurrent rearrangements of *TP53*, *RB1*, *MDM2*, and *CDKN2A*, as well as *PMP22–ELOVL5* gene fusions [2–5]. The most frequent *TP53* rearrangements (e.g., *TP53–VAV1*, *TP53–EMR1*, *TP53–PPRAD*, and *TP53–KPNA3*) resulted in the inactivation of p53 in osteosarcoma, explaining how *TP53* gene function can be consistently disrupted in osteosarcoma despite the low observed prevalence of *TP53* mutations in sporadic osteosarcomas, as shown by traditional mutation analyses [5,7]. A study of transcriptome analysis on untreated clinical osteosarcoma samples revealed that two other osteosarcoma-specific fusion gene products, *LRP1–SNRNP25* and *KCNMB4–CCND3*, are associated with osteosarcoma cell motility [8], and that a *TP53–KPNA3* translocation is associated with chemotherapy resistance and metastasis [7]. These findings have not been validated in large studies because osteosarcoma-associated gene fusions are not as common as other sarcoma-associated gene fusions (e.g., *EWSR1–FLI1* in Ewing sarcoma [9]). Therefore, further studies will be necessary to validate the role of these fusion genes in osteosarcomagenesis and to identify potential therapeutic targets.

Cellular Origins of Osteosarcoma

There are two primary competing hypotheses regarding the cellular origin of osteosarcoma, the mesenchymal stem cell (MSC) origin hypothesis and the osteoblast origin hypothesis [10–13]. The MSC hypothesis proposes that a mutation-carrying MSC will give rise to osteosarcoma [11,13]. A high frequency of pathogenic variants in the *TP53* and *RB1* tumor-suppressor genes and in the *MYC* and *RAS* oncogenes is found in genomic studies of human osteosarcoma [2,3]. Moreover, transformed human MSCs engineered to deplete RB1 and overexpress MYC – a combination observed in patients with poor survival – acquire malignant osteosarcoma-like properties. These MSCs express osteosarcoma markers CD99, ALP, osteonectin, and osteocalcin (also known as bone γ-carboxyglutamic acid-containing protein, BGLAP) and generate

Glossary

Autophagosome: a double-layered membranous structure formed during the process of autophagy. By fusing with the lysosome, the cell can clear unnecessary or dysfunctional components.

Autophagy: a normal destructive mechanism of the cell to clean up dysfunctional components and recycle usable materials.

Base excision repair (BER): a process that removes DNA base lesions induced by oxidation, deamination, and alkylation.

Calvaria: plural of calvarium, the upper portion of the skull composed of the occipital, frontal, and parietal bones that cover the cranial cavity containing the brain, excluding the jaw and facial regions.

Chromothripsis: extensive chromosomal rearrangements that occur in one or a few chromosomes. This chromosomal patchwork pattern leads to genomic chaos.

Clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9): genome-editing methodology built from guide RNA (gRNA)-conjugated DNA nuclease or nickase. The CRISPR/Cas9 system borrows from the bacterial immune system that defends against foreign genetic elements to produce CRISPR RNA (crRNA) based on foreign RNA material. The CRISPR/Cas9 system comprises a gRNA for recognition, and Cas9 for cleavage. The Cas/crRNA complex can target and cut DNA at an arbitrary site based on base-pairing to complementary RNA. Currently, this system is the most convenient methodology for genome editing, although the more accurate but limited paired CRISPR/Cas9 nickase system is also available to target genomic loci. Cas9 will not successfully bind and cleave target DNA regions unless they contain the $^{5'}$NGG$^{3'}$ protospacer adjacent motif (PAM) sequence.

Copy-number alterations (CNAs): some genes are duplicated or deleted in the genome. A difference in the duplication number of a repeated genome area defines its copy-number alteration.

DNA double-strand (ds) break repair (DSBR): the process that repairs DNA with breaks in both strands.

Embryonic stem cells (ESCs): cells isolated from the inner cell mass

Box 3. Rothmund–Thomson Syndrome (RTS) and RAPADILINO Syndrome (RAPA)

RTS is an autosomal recessive genetic disease characterized by a distinct skin rash called poikiloderma that typically develops between the age of 3 and 6 months [60]. The common clinical findings in RTS patients include poikiloderma, small stature, skeletal dysplasia, radial ray defect, sparse scalp hair, sparse brows/lashes, cataracts, skin cancer, and osteosarcoma [145]. Recently, RTS has been categorized into two types, I and II, based on the absence (type I) or presence (type II) of mutations in the *RECQL4* gene [142]. Notably, osteosarcoma is noted in 32% type II RTS patients but not in type I RTS patients [142]. RECQL4 is a member of the RECQ DNA helicase family, which also includes the WRN and BLM proteins that are associated with WS and BS, respectively [143,151]. The majority of patients with type II RTS are compound heterozygous for *RECQL4* mutations, and most of these mutations are predicted to generate a truncated protein. Genotype–phenotype correlations have shown that type II RTS patients are at much higher risk for skeletal defects and osteosarcoma compared to type I RTS patients [60,142,144,145].

RAPA is an autosomal recessive congenital disorder first reported in 1989 in the Finnish population [146]. RAPADILINO is an acronym for the disease features (RA, radial hypoplasia/aplasia; PA, patellar and palate abnormalities; DI, diarrhea and dislocated joints; LI, little size and limb malformation; NO, slender nose and normal intelligence [146]). Although RAPA patients have mutations in the same gene, *RECQL4*, as RTS patients, they lack poikiloderma which is the hallmark feature of RTS [147]. However, similarly to RTS, RAPA patients also show a high incidence of osteosarcoma and lymphoma, with two and four cases, respectively, being reported within a cohort of 15 RAPA patients [148].

lung and liver metastases in immunocompromised mice, suggesting that MSCs constitute the cellular origin of osteosarcomas [14].

By contrast, the osteoblast origin hypothesis suggests that osteosarcoma arises from defective differentiation of osteoblast-committed cells (Figure 1). This hypothesis stems from studies of MSCs derived from *Trp53*-mutant mice showing that a *Trp53* mutation might result in early osteogenesis but impedes final maturation from osteoblast precursors into mature osteoblasts, which is evaluated by the expression of early and intermediate osteogenic marker osteopontin, rather than the terminal osteogenic marker osteocalcin [12]. Moreover, during osteogenic differentiation, depletion of *Trp53 or both Trp53* and *Rb1* in murine bone marrow-derived MSCs (BM-MSCs) – but not in adipose-derived MSCs (ASCs) – induced the formation of osteosarcoma-like tumors [15]. Both undifferentiated BM-MSCs and ASCs developed **leiomyosarcoma-like tumors** but not osteosarcoma. This finding emphasizes that osteogenic differentiation of MSCs is crucial for osteosarcoma development [15]. iPSC–derived osteoblasts, but not MSCs, obtained from LFS patients maintained *in vitro* and *in vivo* tumorigenesis, as evidenced by anchorage-independent growth (AIG) assays and xenotransplantation into immunocompromised nude mice, respectively [10]. This suggests that osteoblasts rather than MSCs are the cells of origin of osteosarcoma. Supporting this notion, RUNX2 and WNT signaling pathways, essential for osteogenic differentiation, have been found to be disrupted in human osteosarcoma samples, demonstrating loss of RUNX2 transcriptional activity and nuclear accumulation of β-catenin, and thus that osteosarcoma development might entail differentiation defects [16,17]. In addition, activation of the intracellular domain of Notch1 in osteoblast-specific conditional Notch intracellular domain (NICD) transgenic mice was shown to promote immature osteoblast proliferation, and was sufficient to induce osteosarcomagenesis [18].

These hypotheses might be at least partially reconciled if the mutation-carrying MSCs indirectly result in osteosarcoma by potentiating the generation of osteoblasts with defective differentiation. Alternatively, given the variability across osteosarcoma tumor samples, both MSCs and osteoblasts might contribute to osteosarcomagenesis. Conditionally disrupted *Trp53* and *Rb1* in murine MSCs, pre-osteoblasts, and mature osteoblasts are all reported to develop into osteosarcoma [19]. Finally, osteosarcoma may arise from mature osteoblasts and osteocytes. For instance, the osteocyte marker dentin matrix acidic phosphoprotein 1 (DMP1) is increased in patient osteosarcoma samples, and SV40-immortalized mouse osteocyte cell lines can

of the blastocyst at the preimplantation stage and cultured. These cells can differentiate into all adult lineages (pluripotency) or proliferate indefinitely without differentiation (self-renewal), based on the environmental conditions.

Epiphysis: bony tissue at the end of a long bone. Before bone growth completes, it is separated from the bone shaft by the growth plate cartilage. After that, it is connected to the bone shaft by ossification of growth plate cartilage.

Epithelial–mesenchymal transition (EMT): the transition of a cancer cell from an epithelial to a mesenchymal morphology, allowing for the movement of the cancer cell into lymph and blood vessels, thereby promoting metastasis. To accomplish this, several genes (e.g., those encoding E-cadherin, SNAIL and TWIST) are alternatively regulated.

Fanconi anemia (FA): a genetic disease that causes bone marrow failure to produce new blood cells and increases the risk of some types of cancer.

Holliday junction: a DNA structure comprising four dsDNA strands that is formed during homologous recombination. Named after Robin Holliday.

Homologous recombination (HR): the exchange of DNA sequences between homologous DNA strands.

Imprinted gene network (IGN): a specific group of imprinted genes with correlated expression. These genes coregulate each other's expression during embryonic growth. The IGN may control a complex regulatory network to induce rapid but controlled developmental processes.

Induced pluripotent stem cells (iPSCs): by introducing defined factors (e.g., the 'Yamanaka factors' OCT4, SOX2, KLF4, and MYC), fully differentiated somatic cells can be reprogrammed into PSCs and gain full differentiation abilities.

Kataegis: a large number of mutations located at particular genomic positions rather than being spread throughout the genome. Kataegis (Greek for 'thunderstorm'), represents the nature of clustering of this mutational thunderstorm. Chromosomal rearrangements are also involved in these regions.

Leiomyosarcoma-like tumors: tumors bearing histological features

Box 4. Werner Syndrome (WS) and Bloom Syndrome (BS)

WS is a rare autosomal recessive genetic disorder characterized by features of accelerated aging after puberty including premature loss of skin elasticity, grey hair, cataracts, diabetes, and cardiovascular disease [114]. Mutations in the DNA helicase *WRN* gene are associated with the development of WS [143]. To date, 83 different mutations spanning the *WRN* gene have been identified in WS patients [65]. Comprehensive genetic investigation of WS patients revealed a higher disease incidence among the Japanese [149]. WS patients are at a higher risk of rare cancers, including soft-tissue sarcoma, osteosarcoma, melanoma, meningioma, thyroid carcinoma, and gastric carcinoma [149]. In a case series of 158 patients with WS from 1996, 12 cases of osteosarcoma were reported [149].

First described by David Bloom in 1954, BS is a rare autosomal recessive disorder. BS is characterized by growth retardation, short stature, malar hypoplasia, hypo- and hyperpigmentation, immune deficiency, infertility, occasional mild mental retardation, and butterfly rash [150,151], and is caused by mutations in the *BLM* gene [77]. Sixty-four different mutations of *BLM* have been reported to date [77]. Among these 64 mutations, 19 were found in more than one patient [77]. The Ashkenazi Jewish population exhibits a higher incidence of BS [152], with ~1% of Ashkenazi Jews being carriers who harbor a heterozygous frameshift mutation in *BLM* [153]. Patients with BS are predisposed to all cancers seen in the general population (such as leukemia, breast, and colon cancers), but the disease onset is much earlier than normal, with an average of diagnosis of cancer at 27 years of age [154]. Two cases of osteosarcoma were reported among 100 cases of cancers found in 168 BS patients in 1997 [155]. Although the incidence of osteosarcoma in BS is not as high as other syndromes predisposing to osteosarcoma, it still far exceeds the expected rate in the general population [156]. BLM functions in preventing recombination by disrupting recombination intermediates *in vitro* [157]. Human BS cells exhibit increased spontaneous chromosomal breaks and increased sister-chromatid exchanges (SCEs), and these cytogenetic features are used clinically to help establish the diagnosis of BS [154].

engraft as tumors in mice via either subcutaneous or intratibial injection [20]. Therefore osteocytes might also constitute an osteosarcoma progenitor cell type. Taken together, although there is still a debate regarding the cellular origins of osteosarcoma, specific genetic alterations may represent key factors in driving the development of osteosarcoma across cell types.

Molecular Mechanisms Involved in Syndrome-Associated Osteosarcomas

Hereditary genetic disorders associated with predisposition to osteosarcoma are relatively rare. However, studies of these diseases have led to important insights that generalize to the broader osteosarcoma population. We discuss here the most recent findings on eight genetic diseases that predispose to the development of osteosarcoma, collectively informing our understanding of the underlying molecular determinants: LFS (Box 1), RB (Box 2), RTS and RAPA (Box 3), WS and BS (Box 4), as well as DBA and PDB (Box 5) (Figure 2).

p53 in LFS

The tumor-suppressor p53 is a transcription factor that binds to DNA as a tetramer and inhibits tumorigenesis by regulating genes involved in cell cycle, DNA damage responses, apoptosis, and metabolism [21]. As a transcription factor, p53 exerts its regulatory effects on thousands of genes regulating hallmarks of cancer [21,22], including angiogenesis, metabolism, cell cycle, apoptosis, **autophagy**, metastasis, and immune surveillance. Various studies have recently demonstrated that p53 plays additional roles in regulating embryonic and somatic stem cell differentiation, and that defects in the p53 signaling pathway lead to impaired cellular differentiation [23,24]. Impaired differentiation may in turn facilitate the development of several types of cancer such as osteosarcoma [25]. While p53 is traditionally thought to primarily promote cellular differentiation, studies on the role of p53 in bone development suggest that p53 might play a negative regulatory role by attenuating osteoblast differentiation [26,27]. Somatic mutations in the *TP53* tumor suppressor gene are one of the most frequently noted alterations in almost every type of cancer, and are found in up to 70% of specimens (http://p53.free.fr/Database/p53_cancer_db.html). The hot-spot sporadic mutations are similar to germline mutations with the exception of the R337 mutation [22].

of leiomyosarcoma during *in vivo* tumor formation. Leiomyosarcoma is a soft-tissue sarcoma arising from smooth muscle cells.

Mitochondrial nucleoids: the complexes formed by mitochondrial DNA (mtDNA) and proteins within mitochondria.

Non-homologous end-joining (NHEJ): the pathway to repair ds breaks by direct ligation of two broken DNA strands. It is an error-prone process.

Nucleotide excision repair (NER): a DNA repair mechanism that eliminates DNA lesions induced by UV irradiation.

Promyelocytic leukemia (PML) nuclear bodies: spherical structures found in the nuclear matrix, are generally composed exclusively of proteins. They play important roles in transcription, apoptosis, and the DNA damage response.

Single-nucleotide variations (SNVs): alterations in the DNA sequence comprising only a single-nucleotide change.

Sister-chromatid exchange (SCE): the breaking and rejoining of DNA sequences between two sister chromatids of one chromosome during DNA replication.

Structural variations (SVs): variation in a DNA region >1 kb in length arising from insertions, deletions, duplications, copy-number alterations, inversions, or translocations that change the structure of the affected region.

Transcription activator-like effector nucleases (TALENs): highly accurate genome-editing methodology that combines the *Fok*I restriction enzyme with transcription activator-like effectors (TALEs). TALEs are derived from *Xanthomonas* bacteria and are built from highly conserved 33–34 amino acid sequences, each of which can recognize a unique base pair. Like ZFNs, target recognition brings *Fok*I to a specific location and permits induction of a ds break.

Variegated translocation mosaicism: a cytogenetic characteristic of Werner syndrome (WS) fibroblasts in which chromosomal rearrangements in cell lines isolated from an individual patient demonstrate a clonal effect.

Whole-genome sequencing (WGS): the process of analyzing the complete DNA sequence of an organism. It provides comprehensive

Box 5. Diamond–Blackfan Anemia (DBA) and Paget's Disease of Bone (PDB)

DBA is a rare inherited disease characterized by red blood cell aplasia with an incidence of around 1:100 000 to 1:200 000 live births. Affected patients frequently have congenital anomalies and display an increased risk of cancer development [158]. About half (~52.9%) of DBA patients carry heterozygous ribosomal protein mutations, and up to nine ribosomal protein genes are reported to be involved in DBA [158]. The most commonly identified mutated gene in DBA is ribosomal protein *S19* (*RPS19*), located at 19q13.2 and accounting for ~25% of DBA [158]. The next most common cause (~7%) is a mutation of *RPL5*, which is associated with a relatively high incidence of developmental anomalies compared with other forms of DBA [96,158]. The specific pathogenesis of DBA is still unclear, although several hypotheses have been offered to explain the anemia phenotype. Owing to an insufficiency of ribosomes for translation, some have proposed that ribosomal defects interfere predominantly with the development of highly proliferated cell populations, such as erythrocytes [159]. Others have suggested that disruption of the heme-exporter FLVCR1 in RPS19-mutated DBA patients may lead to heme toxicity and apoptosis of erythroid progenitors [160]. In addition, ribosomal protein haploinsufficiency leading to a reduced translation of GATA1, a crucial hematopoietic transcription factor for erythrocyte maturation, has been found in DBA patients [161]. Finally, a DBA zebrafish model reveals that defects in ribosome synthesis accelerate apoptotic death of hematopoietic progenitors as a result of p53 activation [92]. DBA patients have an unexpectedly high incidence of osteosarcoma, with six affected patients among 608 in the DBA Registry of North America [162,163]. To date there is no defined explanation for the high incidence of osteosarcoma in DBA patients.

PDB, first described by James Paget in 1876, is a focal disorder of bone turnover causing enlarged and disorganized bones [164]. The disease affects 1–2% of the general population and is more common in the elderly and in males [164]. Approximately 1 in 650 PDB patients develop osteosarcoma, a 30-fold increased incidence compared with the general population over 40 years [164]. The number, size, and the sensitivity to growth factors of osteoclasts in PDB is increased, resulting in abnormal bone homeostasis and the development of skeletal deformities [165]. This rapid bone turnover due to increases in both osteoclastogenesis and osteoblastogenesis may be the cause of increased incidence of osteosarcoma [164]. This turnover hypothesis may also explain why adolescents are particularly prone to development of osteosarcoma due to increased osteoblastogenesis between the ages of 10 and 19 years. The most frequent germline-mutated gene in both inherited and sporadic Paget's disease is *SQSTM1* [165], a gene involved in the RANK signaling pathway and in autophagy. The similarity of cytokine profiles between osteoimmunological diseases (e.g., osteoporosis) and PDB as well as evidence of increased interferon-mediated signaling in PDB [166] suggest that PDB may be a potential osteoimmunological disorder.

genetic information and can be used to identify all variations from a reference genome.

Zinc-finger nuclease (ZFNs): this genome-editing methodology employs *Fok*I restriction enzyme-conjugated zinc-finger proteins. Two zinc-finger proteins, each targeted to a specific strand of DNA in opposite directions, work together to define the targeting site. The *Fok*I restriction enzyme domains, brought together by the zinc-finger domains, function only as a dimer, allowing sequence and orientation specificity to generate a ds break and facilitate homologous recombination.

p53 has been shown to suppress tumor angiogenesis and proliferation in osteosarcoma by inhibiting the PI3K–AKT–mTOR pathway [28], further supporting that detrimental mutations in the *TP53* gene can affect other pathways involved in cancer. The PI3K–AKT–mTOR pathway has been identified as an osteosarcoma driver by a sleeping beauty (SB) transposon-based forward genetic screen which introduces mutations into the genome [29]. Importantly, most LFS mouse models recapitulate human osteosarcoma susceptibility [30–33]. Specifically, introduction of mutant p53(R172H) into mice could lead to formation of osteosarcoma [30]. Functional inactivation of the tumor-suppressor function of transactivation (TA) forms of p63 and p73 resulted in increased cell-transforming activity and reinitiation of DNA synthesis [30]. In addition, a LFS iPSC disease model of osteosarcoma revealed that impaired H19-mediated osteoblastic differentiation and tumor suppression were involved in mutant p53-associated oncogenic ability [10].

Gain-of-function mutations in p53 are thought to participate in multiple pathological activities. For instance, mutant p53 is capable of cooperating with the SWI/SNF chromatin remodeling complex to regulate tumor angiogenesis in 2D and 3D cultures of MDA-468 breast cancer cell lines [34]. It also interacts with ETS2 to bind to and upregulate chromatin regulatory proteins including MLL1, MLL2, and MOZ to promote human breast cancer cell proliferation [35]. In addition to regulating chromatin function, mutant p53 associates with SREBP to abnormally upregulate the mevalonate pathway, resulting in disruption of normal acinar structures in human breast cancers [36]. Mutant p53 also upregulates PDGFRβ by inhibiting the p73/NF-Y complex, leading to further invasion and metastasis in a pancreatic cancer mouse model [37]. The effects of these gain-of-function mutations have not yet been characterized in LFS-associated osteosarcomagenesis, and systematic studies of mutant p53 will be important in understanding LFS-associated osteosarcoma etiology.

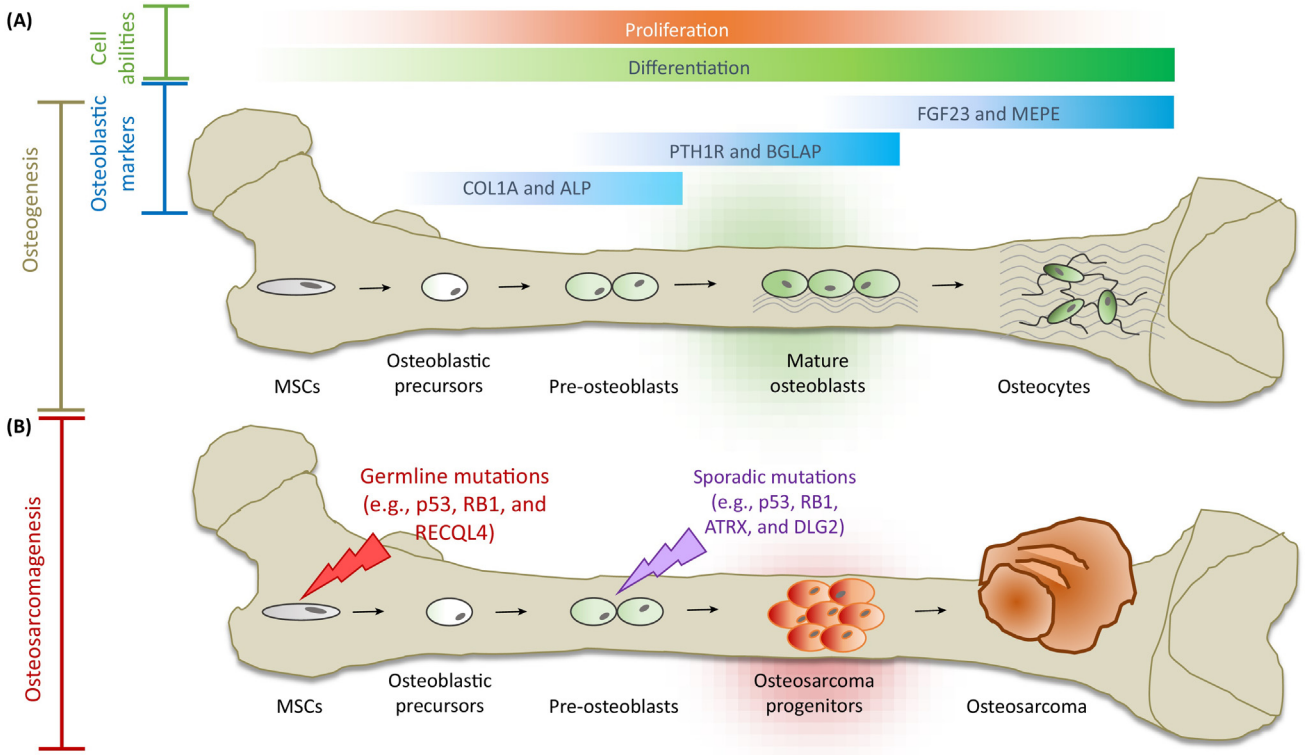
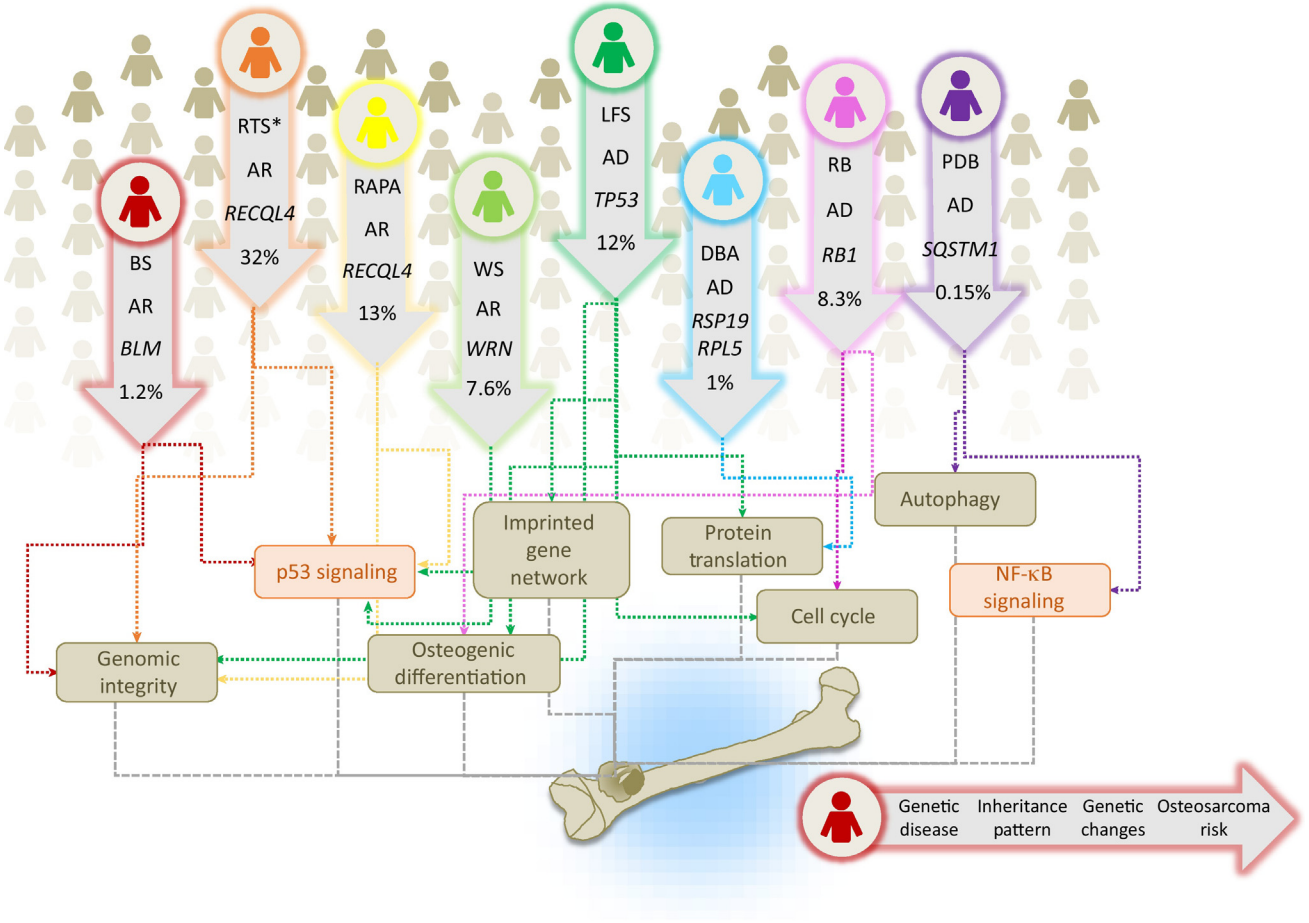


Figure 1. Osteogenesis and Osteosarcomagenesis. (A) Initiation of osteogenic differentiation from mesenchymal stem cells (MSCs). MSCs are multipotent bone marrow cells that are capable of differentiating to bone (osteoblast/osteocyte), fat (adipocyte), and cartilage (chondrocyte) tissues. Osteogenic differentiation is a tightly regulated process involving various signal transduction pathways (e.g., BMP and WNT), transcriptional regulators (e.g., p53, ZEB1, RUNX2, and ZNF521) and cell-cycle controllers (e.g., RB1). Gene expression continuously changes through distinct osteogenic differentiation stages. COL1A and ALP are markers for osteoblastic progenitors and pre-osteoblasts. PTH1R and BGLAP serve as markers for mature osteoblasts. FGF23 and MEPE are markers for osteocytes. (B) Defects in osteogenesis lead to osteosarcomagenesis. Genetic alterations (e.g., germline mutations in *TP53*, *RB1*, and *RECQL4*) probably interfere with the normal osteogenic process, resulting in incompletely differentiated osteoblasts or osteocytes in bone. These defects disrupt the balance between proliferation and differentiation, and may cause a group of cells to display uncontrolled cell proliferation. Osteosarcoma progenitors may arise from these cells and expand to form osteosarcoma.

RB1 in RB

The *RB* gene family, also known as pocket proteins, includes three members, *RB1* (pRB or p105), *RBL1* (p107), and *RBL2* (p130) [38]. *RB1* is located at human chromosome 13q14 and encodes the 110 kDa RB protein (RB1) which mainly participates in negative regulation of cell-cycle progression [39]. In the G1 phase, RB1 is active and binds to E2F transcription factors, inhibiting the expression of both cell-cycle and apoptotic genes [39]. During the G1–S phase transition, RB1 is inactivated via phosphorylation by CDKs. Phosphorylated RB1 releases E2Fs, allowing transcription and cell proliferation to proceed [39]. When RB1 is lost or inactivated by hyperphosphorylation, as commonly occurs in cancer, the cell maintains high expression levels of cell-cycle genes [39]. However, only when RB1 is lost, whether by gene deletion, mutation, or cleavage by caspases, can apoptotic genes be turned on [39]. In such cases and across species, the abrogation of the p53 proapoptotic pathway is required to protect cells from apoptosis [39]. Although expression of genes downstream of E2Fs leads to tumors, the lack of E2Fs also stimulates tumorigenesis in various species [40]. This finding not only implies the essential role of E2F in maintaining cell homeostasis, but also highlights the complexity of the RB–E2F pathway in cell proliferation.



Trends in Molecular Medicine

Figure 2. Familial Syndromes and Osteosarcoma. A cluster of familial syndromes predispose patients to the development of osteosarcomas and are of relevance to understanding the underlying genetics of these tumors. These include LFS, RB, RTS, RAPA, WS, BS, DBA, and PDB. Each inherited syndrome harbors distinct gene mutations but shares a cancer predisposition to osteosarcoma. The dysregulation of variant biological processes (e.g., imprinted gene network, osteogenic differentiation, genomic integrity, protein translation, cell cycle and autophagy) and signaling (e.g., p53 and NF-κB) contributes to the syndrome-associated osteosarcomagenesis. The syndrome-associated risk of osteosarcoma is stated as percentage of patients with disorder developing osteosarcoma. Abbreviations: AD, autosomal dominant; AR, autosomal recessive; BS, Bloom syndrome; DBS, Diamond–Blackfan anemia; LFS, Li–Fraumeni syndrome; PDB, Paget's disease of bone; RAPA, RAPADILINO syndrome; RB, hereditary retinoblastoma; RTS, Rothmund–Thomson syndrome; *RTS, RTS type II; WS, Werner syndrome.

RB1 also plays roles in the progression and metastasis of cancer by regulating angiogenesis and the **epithelial–mesenchymal transition** (EMT) [41]. Moreover, the self-renewal and differentiation properties of **embryonic stem cells** (ESCs)/cancer stem cells are controlled by the RB–E2F pathway [41]. Across different systems, RB1 can regulate differentiation by controlling lineage-specific transcription factors involved in erythrogenesis, myogenesis, cardiogenesis, adipogenesis, and osteogenesis [41,42]. In addition, RB1 can interact with RUNX2 and peroxisome proliferator-activated receptor γ subunit (PPAR-γ) to control osteogenesis and adipogenesis in MSCs, respectively, as shown for different species [16,43]. In addition, the expression levels of RB1 can direct the cell fate of human MSCs toward either the osteoblast/osteocyte or adipocyte lineages [16]. Rb1 expression induces osteogenic lineage differentiation of mouse MSCs, which can differentiate into adipocytes in its absence [44]. Taken together, the role of RB1 in regulating osteoblast differentiation may explain the high incidence of osteosarcoma development in RB patients due to loss of RB1.

RECQL4 in RTS and RAPA

RECQL4 belongs to a member of the RecQ DNA helicase family. Its function has been demonstrated in initiation of DNA replication, DNA damage repair, and maintenance of the integrity of telomeric and mitochondrial DNA [45]. The N-terminus (1–200 aa) of human RECQL4 shares homology with yeast Sld2 protein which is important for initiation of DNA replication [46,47]. Human RECQL4 interacts with DNA replication licensing factor MCM10 to mediate the formation of the CMG (Cdc45, Mcm2-7, GINS) replication complex [48–50]. Because replication stress causes chromosomal instability in human cells [51], mutations in *RECQL4* could cause replication stress leading to genome instability. In addition, RECQL4 directly participates in DNA damage repair, including **nucleotide excision repair** (NER) for UV DNA damage**, base excision repair** (BER) for oxidative DNA damage, and **DNA double-strand (ds) break repair** (DSBR) through **homologous recombination** (HR) and **non-homologous end-joining** (NHEJ) pathways [45]. RECQL4 colocalizes and interacts with xeroderma pigmentosum group A (XPA) protein which is required for NER, and UV damage to H1299 and HeLa cells has resulted in increased co-immunoprecipitation intensity and colocalization between RECQL4 and XPA [52]. In response to H_2O_2-induced oxidative stress, RECQL4 was demonstrated to colocalize with and stimulate the biochemical activities of apurinic/apyrimidinic endodeoxyribonuclease 1 (APE1), DNA polymerase β, and flap structure-specific endonuclease 1 (FEN1), several key factors in the BER pathway, indicating that RECQL4 plays a role in BER in mammalian cells [53]. RECQL4 has also been shown to play a role in NHEJ-dependent DSBR by interacting with and stimulating the activity of the Ku heterodimer, an important member of NHEJ pathway [54]. RECQL4 interacts with p53 and masks the p53 nuclear localization signal, which in turn facilitates p53 mitochondrial localization in untreated normal human fibroblasts [55], providing a new regulatory mechanism of p53 activity. In **mitochondrial nucleoids**, the RECQL4–p53 complex physically interacts with mitochondrial DNA polymerase (PolyA/B2) in human fibroblasts and potentiates its binding to the mitochondrial DNA (mtDNA) control region (D-loop), as demonstrated by electrophoretic mobility shift assays [56]. In addition, RECQL4 can be recruited to laser-induced ds breaks (DSB) by the MRE11 nuclease in the human osteosarcoma U2OS cell line, and is required for 5′-end resection of DSB, the initial step of HR-dependent DSBR [57]. In mammalian cells, RECQL4 also associates with RAD51, a key protein involved in HR; thus, the defect in RECQL4 function is expected to result in defective HR-associated genomic instability [58]. Supporting this idea, karyotype analyses in cells from *Recql4*-deficient mice show increased aneuploidy and premature centromere separation [59]. Therefore, it is possible that defective HR-induced genomic instability might contribute to initiating osteosarcoma development in RTS patients with *RECQL4* mutations. Indeed, although the molecular mechanism of osteosarcomagenesis in RTS remains unclear, genomic instability due to mutations in *RECQL4* has been implicated in disease development. For instance, *RECQL4* is also mutated in RAPA, which also presents an increased risk of osteosarcomagenesis [60].

While global knockout of *Recql4* (targeting exons 5–8) in mouse causes embryonic lethality [61], inactivation of *Recql4* in skeletal tissues has recapitulated some features of RTS, including skeletal abnormalities and small stature [62]. Cells from such *Recql4* conditional mutant mice display elevated p53 activity [62], and the skeletal phenotypes in these mice can be partially rescued by p53 inactivation [62]. Furthermore, mice that lack *Recql4* in osteoblast progenitor cells demonstrate a decrease in mineral apposition rate and bone formation rate [63] but no increase in osteosarcoma. These results imply that osteosarcoma susceptibility is most likely triggered by mutant, not null, alleles of *RECQL4* in RTS patients.

WRN in WS

The human WRN protein contains a conserved 3′ to 5′ helicase domain as well as a RecQ helicase conserved (RQC) region. The helicase, RNase D, C-terminal conserved (HRDC) region

in WRN is also shared by BLM. WRN is the only RecQ helicase member to also possess 3′ to 5′ DNA exonucleolytic activity [64]. WRN can also unwind unusual G quadruplex (G4) DNA structures and four-way junctions [65]. WRN phosphorylation at S1133 by CDK1 favors WRN/DNA2 (DNA replication helicase/nuclease 2)-dependent long-range DNA resection and promotes homologous recombination in human fibroblasts [66]. WRN can also interact with HDAC1 and 2 to promote replication restart following replication stress in mammalian cells [67]. Although loss of WRN in WS may directly lead to cancer development through increased chromosome instability [68], its overall function in cancer is not clear. WRN has been reported to be upregulated in several types of cancer. The ability of WRN to stabilize chromosomes (e.g., BER, proofreading, resolution of G4 for proper DNA transcription and telomere stabilization) may benefit cancer cells with higher rates of proliferation and associated errors [69]. In addition to maintaining genomic integrity, WRN can interact with the C-terminus of p53 through its C-terminal region [70]. Moreover, overexpression of WRN can enhance the transcriptional activity of p53 which leads to increased $p21^{WAF1}$ protein expression in mammalian cells [70]. p38 MAPK activation leads to accelerated cell senescence in WS fibroblasts, implying a potential therapeutic role for a p38 inhibitor in WS [71,72]. Because vitamin C supplementation increased the lifespan of *Wrn*-mutated mice and *C. elegans* [73,74], vitamin C has been reported to be a potential new treatment for WS [75,76]. However, the effects of these compounds on WS-associated osteosarcoma and/or osteosarcoma with defects in WRN-associated signaling have not yet been explored.

BLM in BS

BLM belongs to the RecQ family of DNA helicases. Numerous mutations in human *BLM* causing premature stop codons have been shown to lead to BS [77]. Like WRN, BLM contains a helicase domain, an RQC domain, a HRDC domain, and a C-terminal nuclear localization sequence [78]. In addition to ATP-dependent 3′ to 5′ DNA helicase activities, BLM can interact with topoisomerase IIIα (TOP3A) and stimulate torsional stress relief during DNA unwinding [79]. BLM and TOP3A cooperatively resolve the double **Holliday junction** and prevent **sister-chromatid exchanges** (SCEs) [80] with help from RecQ-mediated genome instability 1 (RMI1) and RMI2. Using biochemical assays, DNA2 has been shown to form a complex with either WRN or BLM and promote DNA repair by facilitating dsDNA degradation [81]. In addition, BLM is involved in chromosome segregation and telomere maintenance in human cells [82,83]. Like WRN, BLM also interacts with the C-terminal domain of p53 and can facilitate p53-induced apoptosis [84]. p53 can help the localization of BLM to **promyelocytic leukemia (PML) nuclear bodies** in mammalian cells [84]. Despite the strong structural similarity between WRN and BLM, only BLM-deficient cells exhibit increased spontaneous chromosomal breaks (BS > WS > WT), sister chromatid exchanges (SCEs), and HR-mediated genomic instability [85], and we speculate that accumulation of genome alterations may induce BS-associated malignancies. By contrast, WS cells are characterized by reduced HR repair and by enhanced mutations and chromosomal aberrations (e.g., **variegated translocation mosaicism** and large chromosomal deletions) [85]. BLM also interacts with several components of the **Fanconi anemia** (FA) gene complex [85]. FA comprises a collection of diseases characterized by mutations in FA pathway genes leading to genomic instability and increased cancer risk. Indeed, BLM can promote FA group D2 (FANCD2) protein activation through its motif VI, indicating that BLM might partially function through FANCD2 in DNA replication [86]. This connection across multiple syndromes highlights the importance of chromosomal instability in cancer [86].

Ribosomal Proteins (RPs) in DBA

DBA is a disorder of ribosome function [87]. RPs are required for normal translation in all cells, and expression of about a quarter of the RPs varies greatly across human tissue types [88]. Hematopoietic cells have among the most heterogeneous RP expression, potentially explaining why DBA has primarily hematologic manifestations [89]. Upregulation of p53 function is

commonly found upon dysregulation of ribosome biogenesis caused by ribosomal abnormalities. Some RPs (e.g., RPL5, RPL11, RPS7, and RPS26) can interact with MDM2 and inhibit E3 ubiquitination of p53, leading to p53 accumulation in both cell lines and mouse model systems [90,91]. Other RPs (e.g., RPS19) have no direct interactions with MDM2/p53, but their mutation nonetheless increases p53 levels in zebrafish [92]. In general, defects in ribosome synthesis accelerate apoptotic cell death of hematopoietic progenitors as a result of p53 activation [93]. Furthermore, ribosomal insufficiency caused by RPS19 and RPS24 mutations can result in G0/G1 arrest and G2/M reduction, respectively, suggesting that cell proliferation defects might contribute to the decreased number of erythroid precursors observed in DBA [94]. The dysregulation of specific RPs has also been noted in cancers [89]. For instance, *RPL5* heterozygous mutations or deletions are found in 11% of glioblastomas, 28% of melanomas, and 34% of breast cancers [95]. Besides being the most common RP mutation in cancer, *RPL5* mutations also lead to craniofacial anomalies [96]. Expression of *RPS19*, the most frequently mutated gene in DBA, is upregulated in several cancers and causes tumor-associated immunosuppression via the complement C5a receptor 1 protein [97,98]. Because of the diverse functions of RPs, the precise mechanisms by which altered ribosome biogenesis in DBA may lead to cancer remains unknown. However, the association between DBA and osteosarcoma suggests an underappreciated role for ribosomal synthesis in regulating and preventing cancer.

p62/SQSTM1 in PDB

p62 (also known as SQSTM1), encoded by the *SQSTM1* gene, is a potent regulator of cell signaling, triggering NF-κB activation, adipogenesis, mTORC1 activation, apoptosis, and selective autophagy through interactions mediated by its different functional domains [99]. When the supply of amino acids is abundant, p62 interacts with regulatory-associated protein of mTOR (RPTOR, also known as RAPTOR) and RAG C/D, leading to p62 translocation into lysosomes, activating mTORC1 signaling [100]. p62 functions by recognizing and binding to selectively ubiquitinated macromolecules: p62 translocates with the ubiquitinated cargo to the lysosome, where it is phosphorylated by mTORC1 at Ser349 [101]. Phosphorylated p62 then causes NRF2 release from KEAP1 and NRF2 stabilization, after which NRF2 can translocate to the nucleus, turning on downstream anti-oxidative genes or forming **autophagosomes** [101]. Interactions between p62 and TRAF6 promote K63 polyubiquitination of mTORC1, which also facilitates mTORC1 activation in both mammalian cells and mouse model systems [102].

Most p62 mutations found in PDB patients occur in the autophagy- and ubiquitin-associated domain [103]. Studies have suggested that these mutations enhance the sensitivity of RANKL and accelerate PDB patient osteoclast formation through NF-κB activation [104]. This signaling pathway might dysregulate the homeostatic crosstalk between osteoclasts and osteoblasts, and promote bone malignancy in PDB as a result of abnormally enhanced osteoclast formation. In addition, accumulation of p62 in epithelial cells, as occurs in pancreatitis and liver degenerative diseases, has been reported to promote tumor initiation and progression [99]. By contrast, p62 is commonly downregulated in tumor stromal fibroblasts; the reduced p62 level decreases mTORC1 activity and MYC levels, ultimately increasing oxidative stress within the tumor microenvironment and promoting cancer progression [99]. It is unknown which of these processes, including autophagy, hyperactivation of RANKL–NF-κB signaling, and changes in the tumor microenvironment contribute to PDB-associated osteosarcoma. Thus, further studies will be necessary to robustly define these associations.

Current Advances in Osteosarcoma Biology Using iPSC Models

In vitro modeling of human disease has recently become possible with iPSC methodologies [105] (Figure 3A). Numerous laboratories have demonstrated that PSCs (ESCs and iPSCs, Box 6) overcome many limitations of other model systems and can serve as a relevant model

system to study the etiologies of cancer including osteosarcoma (in LFS [10], WS [106,107], DBA [108,109], and RB [110]) (Figure 3B), brain tumors [111,112], and leukemia [113].

Malignancy in the LFS iPSC Disease Model

Osteosarcoma is one of the main cancer types seen in LFS patients. An LFS disease model established from patient-derived iPSCs delineated the pathological mechanisms caused by heterozygous p53 (G245D) mutation in osteosarcoma [10]. LFS iPSC-derived osteoblasts recapitulate clinical osteosarcoma features including defective osteoblastic differentiation and gain of tumorigenic ability [10]. Gene expression patterns of LFS osteoblasts are similar to those of tumor samples obtained from primary osteosarcoma patients, and these tumorigenic features strongly correlate with shorter tumor recurrence times and poorer patient survival rates [10].

The numerous chromosomal alterations and rearrangements present in osteosarcoma make analyses of the initial steps of osteosarcoma development particularly challenging in most model systems; however, LFS-derived osteoblasts lack cytogenetic rearrangements and therefore permit the study of early tumor initiation without interference from other gene alterations [10]. Analyses of the global transcriptome in LFS osteoblasts identified impaired expression of the long noncoding RNA H19; functional studies concluded that H19 is an

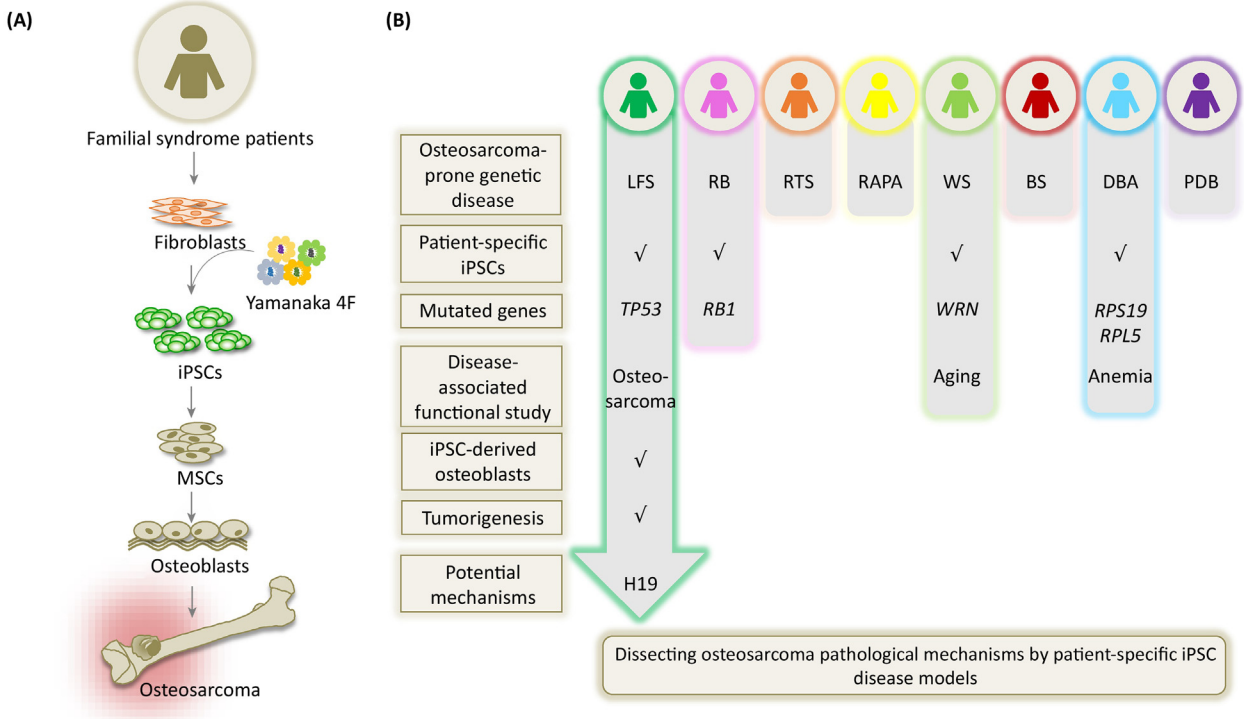


Figure 3. Patient-Derived Induced Pluripotent Stem Cell (iPSC) Disease Models for Osteosarcoma. (A) Patient-derived iPSCs are used to model human familial cancer syndromes and reveal a role of the mutant gene(s) in disease development. To apply iPSC methodology to study genetic disease-associated bone malignancy, patient fibroblasts are biopsied from skin and then reprogrammed to iPSCs by the four 'Yamanaka factors' (YF: OCT4, SOX2, KLF4, and MYC). iPSCs are then differentiated into mesenchymal stem cell (MSCs), and then further to osteoblasts. These iPSC-derived osteoblasts can be examined for osteoblast differentiation defects and tumorigenic ability. Systematic comparison of the genome/transcriptome/interactome between mutant and wild-type osteoblasts can further elucidate pathological mechanisms. (B) Current progress of applying LFS, RB, RTS, RAPA, WS, BS, DBA, and PDB patient-derived iPSCs to model disease etiology and dissect disease-associated osteosarcomas.

Box 6. Modeling Diseases with Patient-Derived iPSCs and ESCs

Characterized by the ability to self-renew indefinitely and differentiate into all cell lineages of an organism similar to ESCs, iPSCs provide a powerful and unlimited source of cells to generate differentiated cells that can be used to elucidate disease pathogenesis, facilitate drug discovery and development, and provide crucial understanding that will be necessary to provide personalized healthcare [125]. Although iPSCs have been used for several years to model diseases such as neurodegeneration, mental retardation, heart disease, and metabolic disorders [125], they have only recently been used for cancer research [126].

iPSCs not only provide unlimited and consistent cell resources but also enhance the applicability of findings by permitting studies within a human model system. Correcting disease mutations in iPSCs by cutting-edge genome editing methodologies (e.g., ZFNs, TALENs, and CRISPR/Cas9) can generate a perfect isogenic control for disease modeling. However, the behavior of each iPSC line must be understood in the context of its genetic background and disease-related specific mutation. A panel of iPSCs, each carrying a distinct genetic alteration in a distinct background, would most generally address the central pathological mechanisms involved in disease development from an affected patient population. However, limited patient samples and the high cost of iPSC generation may restrict this approach. The incorporation of engineered ESCs may help to compensate for this limitation by controlling for genetic background. Various disease-associated mutations can be engineered into the same ESC line, permitting comparison across different disease mutations to understand central disease pathogenesis. Both patient-derived iPSCs and ESCs will aid our understanding of disease pathogenesis and complement existing animal models.

essential gene for normal osteogenesis and tumor suppression, and acts by regulating bone development-associated genes and the **imprinted gene network** (IGN) [10]. This LFS iPSC disease model highlights a previously unappreciated role of p53 in regulating the human IGN that culminates in osteogenic differentiation defects and osteosarcomagenesis [10].

Premature Aging in the WS iPSC Disease Model

Premature cellular aging, telomere dysfunction, and early cell senescence are commonly found in WS patient mesenchyme-derived tissues [114]. One study reprogrammed WS patient fibroblasts to iPSCs and then differentiated these iPSCs to MSCs [107]. Premature senescence was observed in WS MSCs, and a role of telomerase in mediating these defects was suggested [107]. In parallel, another study addressed the effects of long-term culture on WS iPSCs, and documented that WS iPSCs maintain normal self-renewal and differentiation abilities throughout 2 years of long-term culture, indicating that somatic reprogramming might suppress premature senescence in WS [106]. The WS iPSC disease model may help further explore the link between aging and senescence in cancer development including osteosarcomagenesis.

Anemia in the DBA iPSC Disease Model

Erythroid hypoplasia is one of the key features of DBA patients. DBA iPSCs from affected patient fibroblasts harboring either heterozygous RPS19 (Q126X) or RPL5 (R23X) mutations have been established [109]. In comparison with wild-type controls, both RPS19-mutated iPSCs and RPL5-mutated iPSCs have been found to exhibit defective ribosome biosynthesis and globally impaired erythroid progenitor differentiation [109]. These findings suggest that the DBA iPSC model might help in exploring the pathological role of dysregulation of ribosome biosynthesis in developing anemia [109]. The same conclusion was also made by another study using distinct DBA patient iPSCs harboring either RPS19 (R94X) or RPL5 (Y16X) [108]. An unbiased chemical screen was performed on DBA iPSC-derived hematopoietic progenitors, and the autophagy-inducer SMER28 was identified as a potential compound that might enhance erythropoiesis and ameliorate the defect in erythroid differentiation [108].

Although DBA iPSCs have not been used to elucidate the role of ribosome biosynthesis in the pathogenesis of osteosarcoma, many of these earlier findings in anemia may translate to osteosarcoma given the essential role of ribosome synthesis in cellular homeostasis. It will be

valuable to explore whether (i) osteoblasts and hematopoietic progenitors in DBA patients share a similar differentiation defect as a result of heterozygous null mutations in ribosome proteins, (ii) defective autophagy in DBA iPSC-derived osteoblasts contributes to osteosarcomagenesis, (iii) SMER28 can be applied to treat and/or prevent DBA-associated osteosarcoma, and (iv) defective ribosome biosynthesis is a general mechanism leading to osteosarcomagenesis. Because dysregulation of ribosome protein function (e.g., RPL5 and RPL11) influences the p53/MDM2 axis [115], this disease model may also help to clarify the complex signaling networks in osteosarcoma.

Early Studies of the RB iPSC Disease Model

iPSCs were recently generated from a RB patient carrying a heterozygous RB1 (S888A) mutation [110]. Although RB iPSCs expressed pluripotency markers and were capable of differentiation to all germ layers *in vivo*, no pathological and/or mechanistic investigations were conducted in this study [110]. Given the presence of RB1 alterations in 30–75% of sporadic osteosarcoma [116], and the lack of success thus far in recapitulating the osteosarcoma phenotype in RB mouse models [117–119], RB iPSC-derived osteoblasts have the potential to substantially improve our understanding of the role of RB1 in osteosarcomagenesis.

Remaining iPSC Disease Models

RTS, RAPA, BS, and PDB patient-specific iPSC disease models have not been yet established. Consequently, the generation of these disease models through iPSC approaches will be of substantial value in investigating the global picture of the molecular mechanisms involved in osteosarcoma development. Comparisons between the dysregulated gene profiles and signaling networks found in these new lines, as well as those described above, will be crucial to understanding the etiology of osteosarcoma.

Concluding Remarks

Multiple inherited syndromes have been linked to osteosarcoma predisposition. Although attempts at studying the genetics of these diseases have provided fruitful results, an understanding of the role of these genes in development of osteosarcoma is still lacking. There have been attempts to model these genetic diseases with various animal model systems, but few are capable of fully capturing the cancer phenotype. Currently, LFS mice carrying R172H and R270H (R175H and R273H in human), as well as humanized p53 knock-in (HUPKI) mice harboring R175H, R248W, R248Q, and R273H, are capable of modeling much of the broad cancer spectrum found in humans and highlight the importance of gain-of-function activity in p53 [30–32,120,121]. Unfortunately, another HUPKI mouse harboring G245S, one of the most commonly inherited familial p53 mutations, shows similar tumor onset and survival compared to p53-null HUPK mice, and fails to recapitulate its dominant negative function *in vivo* [32]. Moreover, although children with germline *RB1* mutations are likely to experience bilateral multifocal RB and increased risk of osteosarcoma [122], mice with a similar disruption of *Rb1* do not develop either retinoblastoma or osteosarcoma [117–119]. RTS patients carrying *RECQL4* mutations commonly develop osteosarcoma, but deletion of *Recql4* in mice only conveys some RTS clinical features, such as skeletal abnormalities, but not osteosarcoma [63,123]. These results highlight the limitations of using mice to model known genetic risk factors for bone malignancy and emphasize differences across species. This lack of osteosarcoma phenotype in most current mouse models suggests the importance of using other complementary models to study genetic predisposition to osteosarcoma.

Progress in precise gene-editing methodologies including zinc-finger nuclease (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) will aid in providing an alternative means for the introduction of a genetic disease trait into human ESCs (hESCs) and wild-type iPSCs,

Box 7. Clinician's Corner

Patient-derived iPSCs offer substantial potential for clinical application. A clearer understanding of the stepwise genetic changes that occur upon osteosarcomagenesis and progression may be utilized for better prediction of risk in patients with genetic syndromes prone to osteosarcoma and risk of recurrence in sporadic osteosarcoma. In addition, PSCs offer a powerful platform for drug discovery and development, aiding in the discovery of targeted treatments for this tumor.

The genome in osteosarcoma exhibits more alterations than almost all other cancers. Combining WGS and whole-exon sequencing (WES) will help reveal the complex genomic evolution of osteosarcoma and point us towards the identity of osteosarcoma-initiating cells.

Patient-derived iPSC disease models can be applied for modeling disease development, elucidating pathological mechanisms, screening effective compounds to treat and/or prevent patient illness, and testing drug toxicity. In the future it may be possible to apply these osteosarcoma predisposition iPSC models in *in vitro* clinical trials to identify potential compounds targeting osteosarcoma. The panels of identified chemicals could be applied for the first-line screening and suggest personalized therapies for affected patients.

Outstanding Questions

How do deficiencies in genes with ostensibly similar functions (e.g., *RECQL4*, *BLM*, and *WRN*) result in different patient phenotypes? Why do all of these phenotypes include osteosarcoma?

What is the relationship between these osteosarcoma-prone genetic diseases? Do these distinct genetic disorders share common genome/transcriptome/interactome profiles that may promote initiation and progression of osteosarcoma?

Can investigation of these osteosarcoma-prone genetic diseases aid in the development of new strategies to treat sporadic osteosarcoma?

Can differentiation defects constitute a trigger to initiate osteosarcoma development? What are the pathological mechanisms (e.g., epigenetic regulation and defective autophagy) involved in osteogenesis defects?

Can patient-derived iPSCs comprehensively recapitulate human diseases in the absence of a physiological microenvironment (i.e., inside the human body)?

particularly when patient samples are difficult to acquire [124]. With these technologies, osteosarcoma-associated gene alterations (gene deletion, amplification, mutation, and gene fusion) can be easily and arbitrarily introduced into hESCs and wild-type iPSCs to permit the study of their roles in osteosarcomagenesis.

While researchers have found applications for PSCs in a broad range of human genetic diseases with either Mendelian or complex inheritance patterns [125], the application of PSCs to cancer research remains in its infancy. Nevertheless, the work of groups that have begun to apply iPSCs to phenocopy cancer, to explore pathological mechanisms, and to screen potential therapeutic compounds [126] highlights the potential of using human PSCs in medical research. These models of cancer pathogenesis overcome previous limitations related to patient sample availability, or to inapplicability of results from animal models or cell lines with inappropriate genetic backgrounds because of species differences. Modeling genetic disorders with an osteosarcoma predisposition has been successfully demonstrated using the LFS iPSC model [10]. We anticipate significant developments stemming from other hereditary PSC disease models in the coming years (see Outstanding Questions and Box 7). A careful study of the diverse mechanisms of osteosarcomagenesis in these cell lines will help to delineate the pathological mechanisms and reveal potential opportunities for treatment of osteosarcoma in both genetic and sporadic cases.

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